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OXIDATION-REDUCTION INDICATOR PATTERNS IN REGENERATION OF THE FORELEG AND TAIL OF TRITURUS TOROSUS LARVAE

C. M. CHILD

School of Biological Sciences, Stanford University, Stanford, Calif.

In various hydroids, *e.g.*, *Corymorpha* and *Hydra*, reconstitution after removal of a part is largely or entirely by redifferentiation of tissues not directly involved in the injury and often at a considerable distance from it. In the planarian some new tissue which may be regarded as a blastema develops at the surface of injury and gives rise to head and posterior end but more or less extensive redifferentiation of other parts at various distances from the region of injury also occurs. In many cases almost the entire isolated piece undergoes reconstitution into some other part. In these organisms a marked increase in rate of oxidation and reduction of indicators appears at the injured surface almost immediately following removal of a part, *e.g.*, by section, and extends a variable short distance into the intact tissues. This is evidently an injury reaction. As reconstitution progresses, the region of redifferentiation also shows increased reactivity to the indicators, in many forms even before morphological differentiation is evident. *Corymorpha* is a particularly good example of such change in physiological condition preceding morphogenesis (Child and Watanabe, 1935; Watanabe, 1935). In the planarian there is also alteration of indicator pattern adjoining the surface of injury as an injury reaction and in other parts during the following reconstitution but the new tissue regenerating from the cut surface shows more activation than the redifferentiating parts (Child, 1948a).

The reconstitutive capacity of legs, tail, gill filaments and balancers in urodele amphibians has long been known. These and many other cases of reconstitution are commonly called regeneration because regeneration appears to be accomplished by development of a blastema on the surface of injury, apparently involving more or less dedifferentiation of cells with proliferation, growth and redifferentiation, usually into the part removed, and apparently with little or no alteration or redifferentiation of other parts. It has seemed of some interest to attempt to determine whether or to what extent alteration of oxidation-reduction indicator pattern is involved in a case of this sort.

MATERIAL AND METHODS

For leg regeneration *Triturus* larvae 12–20 mm. in length were used, in most cases larvae in which one or more toes were developing on the foreleg. Legs were

amputated at various levels. For tail regeneration larvae ranging from 10 to 16–18 mm. were used with different levels of amputation. The experimental larvae were fed every 2–3 days on pieces of enchytraeids. In stocks not sufficiently fed and with more than a single individual in a container legs were frequently bitten off, usually near the foot. These provided incidental material for stages of regeneration, though time of amputation was not known. These larval stages were used because the regeneration was rapid and because pigment development was not sufficient to obscure indicator patterns.

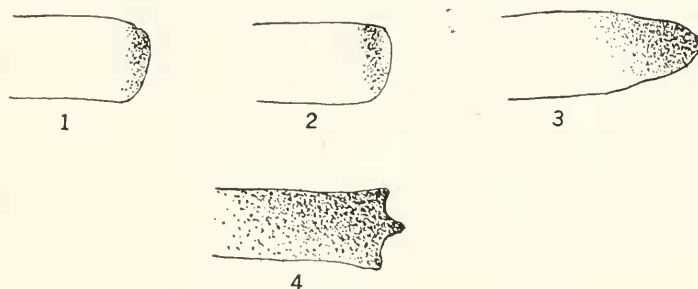
Intracellular formation and reduction of indophenol and intracellular oxidation of the dyes Janus green (diazine green) and methylene blue in solutions reduced by sodium hydrosulphite were used for intracellular oxidations. Following intracellular oxidation (primary oxidation) intracellular reduction of indophenol was obtained by addition of sodium hydrosulphite and of the dyes by addition of more hydrosulphite after primary oxidation. The indophenol reaction was used with very much lower concentrations of the reagents, para-aminodimethyl aniline (dimethyl para-phenylene diamine) and α -naphthol than in the original Nadi reaction. Use of the extremely low concentrations of the indophenol reagents has been described at length in other papers (*e.g.*, Child, 1948a; 1951). The intracellular reaction occurs in a very wide range of low concentrations of the reagents and with the very low concentrations it becomes an exceedingly sensitive indicator of certain differentials in physiological conditions and their changes in living material without appreciable injury but the most satisfactory concentrations for any particular material have been determined by trial. In the present cases, rather rapid reaction being desired, aniline 1/8000–1/16000, assuming that the sample is pure, and naphthol in much lower unknown concentration were used. Enough of the naphthol dissolves in water without heating and without addition of NaOH to give the reaction readily but amount of naphthol dissolving and actual concentrations used are unknown. Differential reduction of intracellular indophenol by sodium hydrosulphite and re-oxidation by oxygen was possible in living material. As a reducing agent sodium hydrosulphite is far superior to the other highly toxic reducing agents used in earlier studies of indicator pattern. Only very small amounts are required for reduction of indophenol or dyes; a small fraction of a milligram in one ml. of water will reduce intracellular indophenol or oxidized dyes in solution or within cells. Hydrosulphite is non-toxic in concentrations much higher than those required for reduction, except that long exposure without re-oxidation may inhibit or kill by lack of oxygen. For intracellular oxidation in reduced dye solution excess of hydrosulphite beyond that required to reduce the dye should be avoided. Excess of the reducing agent may prevent intracellular oxidation. Patterns of intracellular indophenol reaction and of dye oxidation in reduced solution and patterns of intracellular indophenol and dye reduction do not differ appreciably, *i.e.*, regions which oxidize more rapidly also reduce more rapidly than others until high intracellular concentrations of indicators retard or completely prevent reduction differentially.

Figures are necessarily diagrammatic attempts to indicate the differentials in indicator patterns following section and during the earlier stages of regeneration of foreleg and tail.

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FORELEG REGENERATION

Almost at once after amputation, at least as soon as the indophenol reaction or primary intracellular oxidation of dyes became visible, color appeared at the tip of the leg stump earlier than in any other part of the body and gradually extended proximally in the stump for a short distance, usually less than a millimeter, in a decreasing gradient. This injury reaction involved not only the surface of the stump but also internal tissues including the bone which sometimes protruded from the stump. Also the anteroposterior and dorsiventral gradient pattern of the leg (Child, 1943b) usually became distinguishable in slightly more rapid color reaction anteriorly and dorsally in the stump. The injury pattern with anteroposterior differential is indicated in Figure 1. The dorsiventral differential is similar. Patterns of indophenol



FIGURES 1-4. Regeneration of the larval foreleg. Figures 1 and 2, injury reaction, as indicated by intracellular indophenol: Figure 1, early injury reaction, the anteroposterior pattern of the leg appears in the greater reactivity of the anterior side, uppermost in the figure; Figure 2, $1\frac{1}{2}$ -2 hours after amputation, intracellular reduction of indophenol at the extreme tip of the stump, without external oxygen decrease. Figure 3, twelve days after amputation just above knee, primary intracellular oxidation of Janus green to red diethyl safranin in solution reduced to colorless by sodium hydrosulphite, longitudinal and anteroposterior gradient patterns of the leg are evident in the regenerate and increased reactivity extends proximal to the level of amputation. Figure 4, sixteen days after amputation just below knee, indophenol reaction, three toes develop almost at the same time, the longitudinal gradient decreases proximally from the tips of the toes, the anteroposterior pattern from the anterior (upper) side of the figure.

reaction and primary intracellular oxidation of dyes are the same. Later more or less reduction of indophenol and dyes often occurred at the tip of the stump without external oxygen decrease and with the same anteroposterior and dorsiventral differential as the oxidase reaction (Fig. 2). It was noted earlier (Child, 1943b) that such secondary differential reduction often occurred, at least distally, in intact developing legs, gill filaments and balancers without external oxygen decrease and in oxidized dye solutions. Its occurrence suggests high oxidase activity. During the first 2-3 days following amputation the injury reaction appeared to decrease slightly in some cases. An injury reaction also occurred adjoining the cut surface of the distal amputated part of the leg with the same anteroposterior and dorsiventral pattern.

During the earlier stages of regeneration the indophenol reaction and primary dye oxidation in reduced solution were more rapid in the regenerating tissue than in more proximal levels of the leg and other regions of the animal. Anteroposterior and dorsiventral gradients were evident in the regenerating tissue, as well as in the more proximal intact parts and the increase in reactivity appeared to extend a short

distance into the intact part of the leg proximal to the level of amputation (Fig. 3). The reduction differentials following the primary oxidative reaction were similar until intracellular concentrations of indophenol or oxidized dyes became so high that reduction was differentially retarded or completely inhibited in the most susceptible regions in which the primary oxidative reaction was most rapid. Under these conditions more or less reversal of the color gradient pattern occurred.

Sixteen days after amputation approximately at the knee early stages of 3 toes were visible in some individuals and the exact boundary between the regenerated tissue and more proximal regions was becoming less distinct. The increased reactivity of the regenerating region was still evident and still appeared to extend somewhat proximal to the level of amputation. In the regenerated region the reactivity decreased proximally from the tips of the toes and continued without sharp boundary into the pattern of the uninjured region (Fig. 4). In this appearance of three toes at almost the same time the regenerating leg differs somewhat from the order of toe development in the uninjured leg. In those legs one toe appears in the dorsal or anterodorsal region, *i.e.*, from the highest levels of the gradient pattern of the leg, a second toe, slightly later from a more ventral level and a third, still later, also dorsal but becoming posterior to the first. In the regenerating leg the two toes developing from the higher gradient levels differ very little in rate or stage of early development and in reactivity. The third toe, uppermost in Figure 4, shows slightly less reactivity. In later stages the middle toe of Figure 4 elongates more rapidly than the others. At the stage of Figure 4 early development of the foot is also becoming evident in the slightly broader distal region of the regenerate. The anteroposterior and dorsiventral differentials in the pattern of the leg become evident progressively from the tips of the toes proximally through the regenerate into the proximal region of the leg. Progress of reduction is in the same direction until toxic effect retards or inhibits reduction distally, first of all in the toes. With rapid oxygen increase in the solution re-oxidation pattern is like the pattern of primary oxidation but with slight or slow oxygen increase is reversed. The superficial tissues of the leg were injured by the indicators earlier than the internal tissues, so that it was possible at a certain stage of toxic effect to observe more or less reversal of the original reduction gradient pattern superficially with persistence of the original pattern in the internal tissues. In later stages of leg regeneration the pattern gradually approaches that of the intact developing leg, as described elsewhere (Child, 1943b), consequently further consideration of the regeneration pattern is regarded as unnecessary.

REGENERATION OF THE TAIL

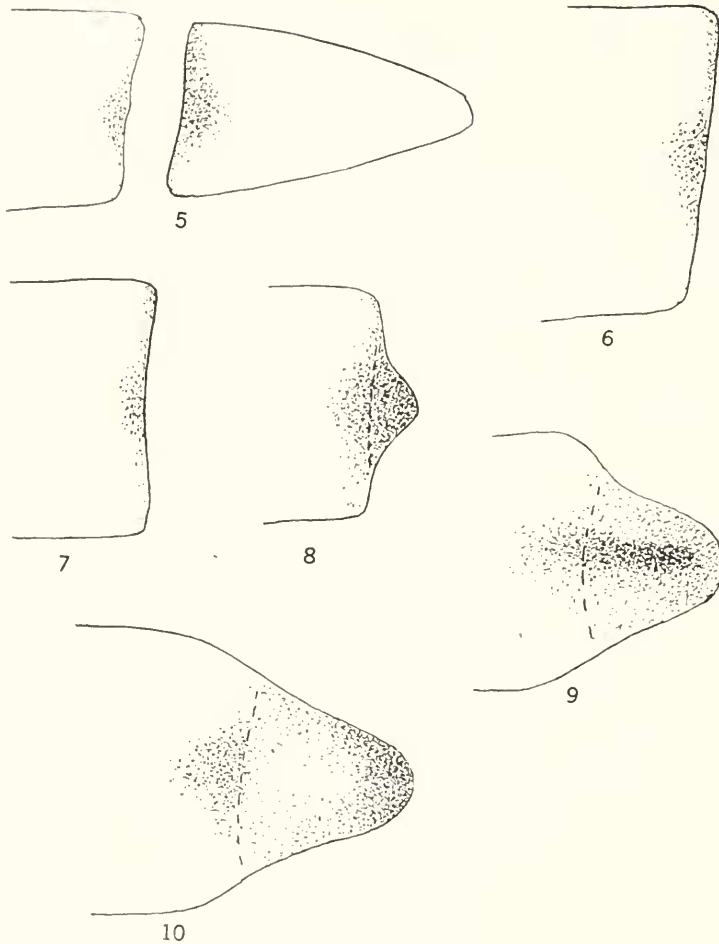
In an earlier study of indicator patterns in early amphibian development (Child, 1948b, pp. 95-99) it was noted that when larval stages earlier than those used in the present paper were completely separated into successive pieces by transverse section at different levels it was found that the reactivity, as indicated by intracellular indophenol, intracellular oxidation of dyes in reduced solution, and intracellular reduction of both indophenol and dyes by hydrosulphite adjoining both anterior and posterior cut surfaces, decreased from anterior to posterior levels of section of the body and tail and from dorsal to ventral at each level of section. In each piece the reaction was more rapid and the spread of the reaction from the cut surface was greater in the posterior direction from the anterior cut surface than in the anterior direction from

the posterior surface and reactivity and spread were greater at the anterior end of each piece than at the posterior end of the piece immediately anterior to it, *i.e.*, at virtually the same body level reactivity and spread were less when the cells concerned were at the posterior end of a piece than when they were at the anterior end of the following piece. In the somewhat later larval stages used as material for tail regeneration transverse sections at various levels of the tail and at more anterior levels show the same differences in the injury reactions with differences in level of body or tail and in anterior or posterior position in the piece as the earlier stages. A single example following section of the tail halfway between the anus and the tip is sufficient to illustrate the point (Fig. 5). This is actually a case of intracellular reduction of oxidized Janus green to the red diethyl safranin, the parts reducing most rapidly being shaded for comparison with figures showing oxidase reactivity. The figure shows the condition 15 minutes after section: reduction to red is more rapid and the spread posteriorly of reduction in the posterior separated piece of the tail is greater than at the posterior end of the tail stump, both in the region of the nervous system and muscles, the "axial" region of the tail and in the dorsal and ventral fins, much less in the fins than in the axial region but slightly greater in the dorsal than in the ventral fin in both the stump and the separated tip. In earlier stages of the reaction a slight gradient, decreasing ventrally, is usually distinguishable in the fins.

Following transverse section at a level approximately one mm. posterior to the tip of the early hindleg the injury reaction is more rapid and extends farther anteriorly in both the body region of the stump and the fins (Fig. 6) than after section at more posterior levels (Fig. 7, also the reaction in the stump of Fig. 5). In short, the relations of these injury reactions to the gradient pattern of the individual are the same in these later larval stages as those previously observed in earlier stages after section at various levels. The injury reaction following section in animals under chloretone anaesthesia appeared to be somewhat less rapid and less extensive than in unanaesthetized larvae. The injury reaction in the tail stump is more rapid than in any other part of the larva.

Figure 8 is an example of indicator pattern in the regenerating tail 7 days after amputation near the middle. There are of course individual variations in rate of outgrowth of tissue other than those resulting from amputation at different levels. The outgrowth is chiefly in the middle region in which the nervous system, spinal cord and muscles develop. Dorsally and ventrally the outgrowth decreases. At this stage the outgrowth shows merely a general gradient decreasing anteriorly without visible distinction of particular parts. It is noted as a point of some interest that the increased reactivity extends into tissues anterior to the level of section with a further decrease anteriorly of the gradient for a distance almost equal to the length of the regenerate; even in this region the reactivity is greater than in other parts of the larva. In general, rate of tail regeneration and to some degree the increase in reactivity in the regenerate and anterior to the level of section decrease as level of section becomes more posterior. Detailed description of such differences seems unnecessary.

In more advanced stages of regeneration individual differences in rates of outgrowth and in conditions in the tissues of the outgrowth appear. For example, in Figure 9, 17 days after amputation, the usual gradient, decreasing anteriorly, appears but the developing axial structures in the regenerate show much greater reactivity than the rest of the outgrowth, also with a gradient decreasing anteriorly.



FIGURES 5-10. Regeneration of the larval tail. Figure 5, injury reaction following transverse section at middle of tail, as indicated by intracellular reduction of Janus green, 1/30,000, to red diethyl safranin after staining by the oxidized dye; rate of reduction is higher than in any other part of the animal and its extent, both in the caudal axial region and in the fins, is greater posterior than anterior to the level of section. The indophenol reaction pattern is the same.

FIGURES 6 and 7 show the difference in rate and extent anteriorly of the injury reaction in caudal axial region and fins, as indicated by indophenol, after section at an anterior tail level (Fig. 6) and a level posterior to the middle of the tail (Fig. 7): reactivity is much greater and extends farther anteriorly in the caudal axial tissues than in the fins.

FIGURE 8. The indophenol reaction gradient 7 days after sections, with slight decrease anteriorly in the regenerate and further decrease in the caudal axial region anterior to the levels of section and to a lesser degree in the fins; reactivity is still greater than elsewhere in the animal.

FIGURE 9. Indophenol reaction 17 days after section; much more rapid reaction in the regenerating caudal axial region with slight decrease anteriorly, than in the regenerating fins dorsal and ventral to it: anterior to the level of section reactivity decreases anteriorly but extends farther than in earlier stages: reactivity in relation to regeneration is still greater than in any other part of the animal.

FIGURE 10. Indophenol reaction in a slightly more rapid regeneration than Figure 9, 16 days after section: after $\frac{1}{2}$ hour the regenerating caudal axial region has undergone secondary

The high reactivity of this region is evidently associated with certain developmental stages of these structures. Dorsal and ventral to this region there is much less rapid reaction in the developing fins. In the axial part of the tail the increase in reactivity extends farther anterior to the level of section than in Figure 8, with decrease in the anterior direction. In another case (Fig. 10) with somewhat longer regenerate 16 days after amputation the axial structures in the regenerate reacted at first much like those in Figure 9 but secondarily reduced indophenol almost completely without external oxygen decrease, suggesting greater dehydrogenase activity than in the case of Figure 9. In the individual of Figure 10 the increased reactivity also extends a considerable distance into the tissues anterior to the level of section but without secondary reduction.

In still later stages of tail regeneration the difference between the regenerate and more anterior levels gradually decreases and disappears as rate of regeneration decreases. Greater reactivity with a gradient decreasing anteriorly, except when secondary reduction occurs, persists longer in the axial region of the tail than in the fins. Since nothing of particular interest as regards indicator patterns has been observed in these later stages it seems unnecessary to describe them at length.

Intracellular reduction of indophenol and dyes shows the same differentials in pattern until differential retardation or inhibition of reduction resulting from toxic effect of indicators, appearing earliest in regions originally of most rapid indicator oxidation, alters or may reverse more or less completely the gradient pattern. Re-oxidation pattern is similar to the pattern of primary oxidation if oxygen increase following reduction is sufficiently rapid. With slow or slight oxygen increase it is the reverse of the primary oxidation pattern.

DISCUSSION

The appearance of the anteroposterior and the dorsiventral differentials in the injury reaction on the foreleg stump is of interest as evidence that the change in condition of the cells concerned in this reaction depends to some degree on their condition preceding amputation. The increase in reactivity is somewhat greater in cells representing higher gradient levels in the intact leg than in those of lower gradient levels, consequently the gradient pattern is not obliterated in the injury reaction or in later regeneration. Either the differences in physiological condition associated with their original position in the pattern of the intact leg persist more or less completely during the increased reactivity and the probable greater or less dedifferentiation characteristic of the regenerating tissue, or else the pattern is induced in them by the differentials of the stump. The presence of the pattern in the injury reaction preceding actual regeneration perhaps indicates its persistence without induction. However, in the later regeneration and differentiation of the regenerated part its relation to the stump and the ingrowth of nerves doubtless plays a part.

The development of three toes almost at the same time in the regenerating leg, rather than in a definite sequence, as in development of the intact leg is probably to be regarded as a consequence of the higher level of metabolic activity in the regenerating

reduction without external oxygen decrease after a primary reaction: a gradient of increased reactivity, decreasing anteriorly, is present anterior to the level of section chiefly in the caudal axial tissues without evidence of reduction: reactivity in relation to regeneration is still greater than in other parts.

leg than in ordinary development which determines decrease in time differences in progress of morphogenesis.

Following transverse section in the tail the great difference in rate and intensity of the injury reaction in the post-anal extension of the neural, spinal and muscular structures, the "axial" region of the tail, and the dorsal and ventral fins is of interest as evidence of a great difference in physiological condition in the two regions. The reaction in the caudal axial region is not only much more rapid but extends much farther anteriorly from the cut surface than the very slight reaction immediately adjoining the cut surface in the fins. However, even in the slight reaction of the fins the dorsiventral gradient is usually distinguishable in the slightly more rapid reaction of the dorsal fin, and not infrequently a decrease in rate of reaction ventrally is visible in both fins.

It is also of particular interest in relation to the general significance of gradient pattern that the injury reaction is not only more rapid in both body region and fins of the posterior separated piece of the tail than in the anterior stump but also spreads farther posteriorly in the posterior piece than anteriorly in the stump. These differences in injury pattern at posterior and anterior cut surfaces are similar to those observed throughout the entire length of earlier larval stages, separated into successive pieces by a series of transverse sections at different levels (Child 1948b, Figs. 16 and 17). These differences are obviously associated with the anteroposterior gradient pattern of the larval body. On the two sides of the same level of section the injury oxidation is more rapid and spreads farther posteriorly down, than anteriorly up the gradient.

An essentially similar relation to gradient pattern appears in many other organisms, either as a feature of reconstititional morphogenesis, or of functional physiological activities in certain organ systems of developing or mature organisms. In isolated pieces of *Hydra* and the planarian the differentials in reactivity to oxidation-reduction indicators in relation to body-level and to distal and proximal, or anterior and posterior cut ends of the pieces are like those in the larva of *Triturus* (Child, 1948a). Moreover, as regards bipolar forms of hydroids it has been known for many years that hydranths or partial hydranths, developing at the aboral ends of bipolar pieces of *Tubularia* stem within certain limits of length, are usually smaller, develop less rapidly, and are often less complete, than those developing from the oral end. Similar relations appear in bipolar forms of *Corymorpha*. Also in bipolar planarian heads the head developing from the posterior cut end is often smaller, develops less rapidly than the anterior head, and may show all degrees of differential structural inhibition. In both the hydroid and planarian bipolar forms the hydranth or head developing with establishment of a new gradient opposed in direction to the original gradient, involves greater changes in organization than does development at the oral or anterior end and may be more or less inhibited by the preëxisting gradient. However, in both hydroid and planarian pieces so short that the longitudinal gradient pattern is almost completely absent and consequently has little or no appreciable effect on development, apical regions or heads at both ends develop at about the same rate and under almost the same conditions as regards gradient pattern and are usually alike or almost alike.¹

¹ As regards the bipolar forms of hydroids see, for example, Child (1907) and earlier literature cited and discussed there; Child and Watanabe (1935); Watanabe (1935); Child (1941; pp. 36-38, 313-321). For planarian bipolar forms see Rustia (1925); Child (1941; pp. 363-364).

The following examples of relations of functional physiological activities to gradient pattern are also of interest as indicating that morphogenetic and functional relations to gradient pattern are not fundamentally different. In the ctenophore plate row conduction of the impulse determining movement of the plates, following local mechanical or other stimulation at any level of the row, progresses much farther down than up the row. Usually it progresses down the gradient present in the row over the entire length of the row below the levels of stimulation but, unless the stimulation is extreme, only over a few plates up the gradient. The mammalian intestine possesses a longitudinal gradient pattern with high end at the pylorus, and, as Alvarez has demonstrated in a series of papers, conduction following a local stimulus at some level progresses much farther, often over the entire length down the gradient, and over a much shorter distance up the gradient. The vertebrate heart also gives evidence of somewhat similar relations (Child, 1941, pp. 327-330 and references cited there).

The much more rapid outgrowth in the caudal axial region than in the fins is what may be expected in view of the evidence of much more rapid metabolism in that region than in the fins. It is also of interest that the increase in reactivity in the axial region anterior to the level of section with a gradient decreasing anteriorly is characteristic, not only of the injury reaction but of regeneration (Figs. 8, 9 and 10). Evidently the change in physiological condition anterior to the surface of section is associated with the regenerative activity of the caudal axial region. In the fins there is also some increase in the spread anteriorly of slightly increased reactivity. It appears that even in regeneration of amphibian leg and tail which are commonly regarded as a regeneration from a blastema developing at the cut surface, the indicators give evidence of a change in physiological condition extending into tissues anterior to the level of amputation. This suggests the possibility that some degree of redifferentiation may be occurring there and that this case is perhaps not fundamentally different from reconstitution in various invertebrates, *e.g.*, planarians, which is only in part a real regeneration.

In earlier indicator studies on amphibian development (Child, 1943a, 1943b) it has been stated that the re-oxidation gradient, following intracellular reduction, is opposite in direction to the gradients of primary oxidation and reduction, *i.e.*, that regions which primarily oxidize and reduce the indicators most rapidly re-oxidize them after reduction least rapidly and that rates of re-oxidation increase as rates of primary oxidation and reduction decrease. In further work with indicators this has been found to be true when external oxygen increase is relatively slow and slight. However, when it is sufficiently rapid and great the re-oxidation pattern is the same as that of primary oxidation. Larval amphibian appendages frequently reduce indophenol and dyes secondarily, *i.e.*, after first oxidizing them, and without external oxygen decrease, suggesting high dehydrogenase activity. In re-oxidation apparently oxygen available, rather than enzyme activity, may determine the re-oxidation differential if oxygen increase is slow or slight because it becomes sufficient to permit intracellular re-oxidation earlier in regions of lower, than in those of higher oxygen uptake. With rapid and great external oxygen increase the differentials in enzyme activity become the factor determining the re-oxidation pattern. In short, two opposed re-oxidation patterns are possible, not only in amphibian development but in other organisms, one determined by external, the other by internal factors.

SUMMARY

1. Amputation of foreleg and tail at various levels is followed almost immediately by increased intracellular indophenol reaction and oxidation of dyes in reduced solution.

2. In the leg the injury reaction and the later regenerative outgrowth show the anteroposterior and dorsiventral patterns of the leg.

3. Development of the first three toes at almost the same time in the course of regeneration of the foot, rather than in the sequence characteristic of the development of the intact leg probably results from the increased metabolism in the regenerating, as compared with the intact leg.

4. In tail regeneration the injury reaction is more rapid and extends much farther anterior to the surface of amputation in the caudal axial region than in dorsal and ventral fins. Also the later regenerative outgrowth is much more rapid in and near the caudal axial region than in the fins. The reactivity gradient decreases anteriorly.

5. The increase in reactivity anterior to the level of tail amputation is present not only during the injury reaction but also during the following regeneration. Its persistence suggests that considerable physiological change may occur in this region, as well as in the regenerative outgrowth.

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